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Evaluating Candidate Pre- and Post-Treatments for Efficacy
Against Percutaneous Sulfur Mustard Exposure at the Medical
Research and Evaluation Facility

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The mouse ear model has been used at the U.S. Army Medical Institute of Chemical Defense (USAMRICD) to assess dermal injury caused by sulfur mustard (HD) and to evaluate potential pre- and posttreatment compounds for efficacy against percutaneous HD injury. In this model, the effects of HD with or without treatment compounds are evaluated macroscopically by edema and microscopically by histopathological changes associated with HD injury. The objectives of Task 95-43 were first to transition this technique to the Battelle Medical Research and Evaluation Facility (MREF), and then to evaluate candidate antivesicant drugs for their efficacy at eliminating or lessening the effects of HD-induced tissue damage. By June 1997 the mouse ear model had been transitioned and validated at the MREF. The MREF has evaluated 334 candidate HD treatment compounds provided by USAMRICD, and 73 passed the initial screening. Of these, 23 were anti-inflammatory agents, 21 were protease inhibitors, 15 were inhibitors of poly-ADP-ribose polymerase, 12 were HD scavengers, and 2 were other classes of compounds. Several of the successful HD scavengers and anti-inflammatory agents were also tested as posttreatments (applied 10 minutes after HD) and showed some efficacy.

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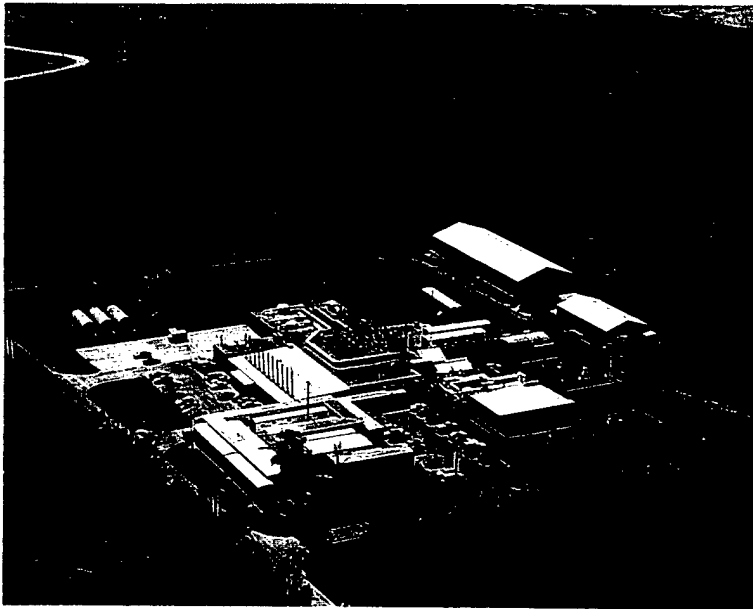
**Task 95-43: Transition and Use of
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Against Percutaneous Sulfur
Mustard Exposure at the Medical
Research and Evaluation Facility**

To

U.S. Army Medical Research and

Materiel Command

April, 2000



FINAL REPORT

**Contract No. DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program**

on

**TASK 95-43:
TRANSITION AND USE OF THE MOUSE EAR MODEL FOR EVALUATING
CANDIDATE PRE- AND POST-TREATMENTS FOR EFFICACY
AGAINST PERCUTANEOUS SULFUR MUSTARD EXPOSURE
AT THE MEDICAL RESEARCH AND EVALUATION FACILITY**

to

**U.S. ARMY MEDICAL RESEARCH
INSTITUTE OF CHEMICAL DEFENSE**

April, 2000

by

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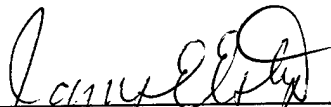
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Executive Summary

The mouse ear model has been successfully transitioned and utilized at Battelle's Medical Research and Evaluation Facility (MREF) since 1997 to evaluate candidate pretreatment and treatment compounds for percutaneous sulfur mustard (HD) exposure. The inflammation (edema) present following percutaneous HD exposure is quantifiable. In addition, tissue is ranked with an incidence and severity score when a histopathologic marker is evident within the affected tissue. Three hundred thirty-four (334) candidate antivesicant drugs were tested topically for their efficacy in protecting against the effects of HD-induced tissue injury.

- Seventy-three of 334 drugs passed at least one end point when tested as pre-treatments and evaluated at 24 hr post-HD challenge.
- Of these 73 drugs: 23 were anti-inflammatory agents, 21 were protease inhibitors, 15 were inhibitors of poly-ADP-ribose polymerase, 12 were HD scavengers, and 2 were other classes of compounds.
- Four were further tested and passed extended protection when tested as pre-treatments and endpoints evaluated at 48 and/or 72 hr post-HD challenge.
- Five were further tested and passed when tested as post-treatments and evaluated at 24 hr post-HD challenge.

Table of Contents

	<u>Page</u>
Executive Summary	i
1.0 Introduction	1
2.0 Experimental Design	1
2.1 Test System	2
2.2 Model Transition	2
2.3 Assessment of Topical Pre- and Post-treatments	3
2.4 Animal Manipulations	5
2.5 Agent Use and Sampling	6
2.6 Biosampling	7
2.7 Study Endpoints	8
2.8 Statistical Analysis	9
3.0 Results	11
4.0 Discussion	11
5.0 Record Archives	13
6.0 Acknowledgements	14

APPENDIX A

Protocol 116

APPENDIX B

Tables

**Task-95-43:
Transition And Use Of The Mouse Ear Model For Evaluating
Candidate Pre- And Post-Treatments For Efficacy
Against Percutaneous Sulfur Mustard Exposure
At The Medical Research And Evaluation Facility**

1.0 Introduction

Sulfur mustard (bis(2-chloroethyl) sulfide; HD) applied to the skin produces no visible immediate reaction, and penetrates rapidly. With time, an erythema (reddening of skin) is observed, followed by fluid accumulation (edema) at the site of contact. Macroscopic skin changes may be delayed for hours, and range from mild erythema to vesication (blistering) and even complete destruction of tissue (necrosis). The mouse ear model is used to assess injury created by HD, with the primary physiological basis being fluid accumulation. The model was developed by investigators at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) to evaluate candidate pre- and post-treatment compounds against percutaneous HD injury. Task Order 95-43 was initiated at Battelle's Medical Research and Evaluation Facility (MREF) in June 1996 to first transition this technology to the MREF, and then to evaluate candidate antivesicant drugs for their efficacy in protecting against the effects of HD-induced tissue injury.

2.0 Experimental Design

MREF Protocol 116 was prepared in June 1996 by Dr. James A. Blank for the conduct of this task. Protocols of animal experiments performed at the MREF were reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC), and by the Animal Use and Review Division of the U.S. Army Medical Research and Materiel Command prior to initiation of a study. Protocol 116 and its amendments are attached as Appendix A. Initially, up to 50 compounds were proposed for evaluation under this protocol. In April 1998, USAMRICD requested that the scope of work for this task be extended to test approximately 240 additional compounds. With testing of 72 candidate compounds having been completed at the time of this request, a total of 312 compounds were targeted for testing under this task of the MREF contract by July 1999.

2.1 Test System

Approximately four-week-old male CD-1 (ICR) outbred mice from the Portage, Michigan facility of Charles River Laboratories were received at the MREF in appropriate numbers to conduct scheduled testing. Upon arrival, mice were group housed (5 per cage) in polycarbonate shoebox cages and quarantined according to facility standard operating procedures. Fluorescent lighting was used with a 12-hr light cycle per day. Room (or hood, if applicable) temperatures were maintained at 64-79 F and the relative humidity was maintained at 30-70 percent. Purina Certified Rodent Chow® was available *ad libitum*, and water was supplied from Battelle's West Jefferson well-water system via bottles. A bio-chamber was utilized frequently for housing during quarantine.

When preparing the animals on a study day, mice weighing in the range of 25 to 35 g were marked for identification, and housed two per cage during pre-treatment application and after HD challenge. Cages were placed on warm water-perfused heating pads within the laboratory biofume hood system overnight following dosing of HD.

The ear of this animal model has been shown by investigators at USAMRICD to exhibit edema as well as microvesication following percutaneous HD exposure. The edema present in HD-exposed mouse ear tissue was determined to be quantifiable by comparing weight measurements of an unexposed mouse ear to the same animal's compound pre-treated, HD-exposed ear. At the cellular level, the incidence and severity of microblisters and necrosis on each side of the HD-exposed ears was ranked with a severity score when a histopathologic marker was present within the affected tissue. During the course of this task from January 1997 through early October 1999, 4,473 mice were used on study to evaluate 334 candidate compounds in this mouse ear model.

2.2 Model Transition

Initially, a HD dose-response study was performed to establish a HD challenge dose that produces a degree of edema similar to that obtained by investigators at USAMRICD for candidate compound evaluations. Mice were exposed to three HD doses and edema was measured at 24 hr following exposure. Each dose group consisted of 10 animals and exposures

were performed as described in a later section. Measurements of edema and histopathology were performed on tissue samples. Statistical analyses were conducted on each HD dose group tested to compare results between experiments conducted at USAMRICD and at the MREF. From these studies, it was determined that there was no significant difference between results from the two laboratories and a HD solution in methylene chloride (32 mg/mL) was selected for dosing a final dose of 0.16 mg contained in 5 μ L.

Next, the successful transition of the model to the MREF was demonstrated by qualitatively duplicating the edema and histopathology results for four compounds that had already been evaluated at USAMRICD.

The four model validation compounds were:

- ICD# 2086: indomethacin
- ICD# 2723: n-vanillyloleamide or olvanil
- ICD# 2842: hydrocortisone
- ICD# 2844: 6- α -methyl prednisolone

The compounds were evaluated as described in the next section. Measurement of edema and histopathology were performed on these samples. Statistical differences obtained at Battelle were qualitatively compared to those obtained at USAMRICD, and it was found that there were no significant differences between the laboratories in the results. By June 1997, the mouse ear model was successfully transitioned and validated at the MREF.

2.3 Assessment of Topical Pre- and Post-treatments

All test compounds were provided to Battelle by the Basic Assessment Branch, Drug Assessment Division, USAMRICD and were submitted to the MREF through the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR). Some of the compounds also were designated for use on other MREF tasks. Compounds were stored in amber desiccator cabinets (or in clear desiccator cabinets if refrigeration was required).

The method for testing a particular compound was assigned by USAMRICD and this information provided to the Study Director. This initially was done with a hard-copy computer printout sent from USAMRICD to the MREF, but was modified in December 1997 so that assignments have been provided via Test Assignment Sheets (TAS) transmitted electronically. In many cases, the solubility of candidate compounds was not known and therefore solubility testing was conducted. If no information on solubility was provided, the first solvent of choice was ethanol, the second acetone, and the third choice dimethylsulfoxide (DMSO). Initially, 10 mg of the compound was weighed into a glass culture tube and 0.5 mL of ethanol added with vortexing. If necessary, sonication and/or warming with hot tap water to help solubilize the compound was performed, if not contraindicated on the TAS. If necessary, more ethanol was added in 0.25 mL increments (with sonicating and/or heating) up to a total of 2 mL. At this point, if the compound was not soluble, another 10 mg was weighed and the same steps taken with acetone, and if still not soluble, with DMSO. The amount of solvent added was recorded and the maximum concentration determined. Most compounds were dosed at the limits of solubility. Inventory records were kept on candidate compound usage.

Each treatment group consisted of 10 animals. A group that did not receive a pre- or post-drug treatment but was exposed to HD was included on each experimental study day. The availability of MREF resources, as well as the backlog of compounds and priority of testing, determined whether only one compound or up to six compounds were tested on each dosing day. Ideally, three compounds were tested along with one control group, on a given dosing day (40 mice).

Candidate compounds (nominally 1 mg) were applied topically to the inner, central part of the right ears of mice. The compound was delivered as a single, approximately 5-10 μ L dose, or was administered in repeated applications to the same site to obtain the total required dose. Up to 30 μ L was applied to the ear surface for any one compound. Calibrated, digital, microliter, positive-displacement, adjustable pipettes were used for applications of compound, methylene chloride, and HD. The time interval for pre- and post-treatment compound application was provided by USAMRICD on the TAS.

Animals were weighed, marked for identification, and anesthetized prior to HD application. Five μ L of methylene chloride was applied to the left ear and five μ L of HD in

methylene chloride at a concentration of 32 mg/mL (195 mM) was applied to the right ear. For HD application, the animals were positioned when anesthetized. The ears were not decontaminated following HD exposure. Following application of HD, the animals were placed two per cage in polycarbonate cages and retained within the fume hood system with food and water for either 24, 48, or 72 hr as assigned by the TAS.

In most cases, approximately 24 hr following HD application, the animals were euthanatized by halothane overdose, the ears cut from the animal, and 8-mm punches of the ear tissues taken. The 8-mm biopsies were weighed, and then placed in a neutral buffered formalin solution for histopathology. Edema and histopathologic changes were determined and statistically evaluated as described in a later section.

2.4 Animal Manipulations

On the day of study, the assigned number of animals plus a few extras, were weighed to the nearest 0.1 g using a calibrated balance (Sartorius model number 3862-MP8-1, Sartorius GmbH, Göttingen, West Germany). Each animal was identified by assigning and writing a number on their tail with a black or red indelible ink marker (black for odd-numbered animals and red for even-numbered animals). Mice were anesthetized by intraperitoneal (i.p.) injection, using a 1-mL tuberculin syringe with a 25 gauge, 5/8" needle, of a 10 mL/kg solution made by mixing 1.0 mL ketamine hydrochloride (100 mg/mL), 1.0 mL xylazine hydrochloride (20 mg/mL), and 15.5 mL of saline. Time of anesthesia was coordinated such that animals were dosed at approximately 2-minute intervals and the time documented. In this fashion, sufficient time was allowed between animals so that tissue sampling could be performed as close to 24 hr following HD dosing. Anesthetized animals were secured in dorsal recumbency on a custom-cut, 3 x 5" tie-down board made of sturdy cardboard. Half-inch wide pieces of cloth tape were used to position the two front limbs and the tail of each mouse on the cardboard.

If assigned as a pre-treatment compound, unanesthetized animals were treated at the TAS-assigned time on the right ear with a specified amount of candidate compound. Compound dilutions were made fresh daily and applied near the hood face. One technician manually restrained the animal and another technician applied the compound to the inner surface of the

right ear. Animals were then returned to their appropriate cages until time for anesthetic injection.

When the mice were anesthetized and positioned on tie-down boards, 5 μ L of methylene chloride was applied to the inner surface of the left ear, at the hood face, approximately 1 min prior to HD challenge. The animal was then moved into the dosing hood where a foam swab was placed under the mouse's head and the head positioned so that the ear being dosed formed a cup that could hold the 5 μ L dose of HD in methylene chloride solution without the fluid running off or into the ear canal. The HD dose was usually applied as three point droplets. Once the methylene chloride evaporated (approximately 20 sec), the animal was released from the tie-down board and placed into its labeled cage and maintained within the fume hood system. Cages contained a small amount of Sani-Chip[®] bedding covered with a piece of absorbent padding. The mice were placed in sternal recumbency with the head positioned so that neither ear was compressed or favored in any fashion. After food and water was placed on each cage top, large absorbent pads were placed over the cages and taped to the sides to help prevent loss of body heat overnight. The hood sashes were kept closed overnight to prevent drafts.

2.5 Agent Use and Sampling

HD dilutions were made approximately every 4 months at a concentration of 32 mg/mL (195 mM) for use on this task. These dilutions were made from neat HD supplied by USAMRICD (U-2325-CTF-N) and usage was recorded in MREF Chemistry Accountability Logbooks 51005, 50016, 50017, and 50018. Aluminum-capped, 1.5-mL vials (glass, amber) were used to store the approximately 1-mL samples of diluted HD in a locked, $< -70^{\circ}$ C freezer. Before dosing, an agent vial was removed from the freezer and placed in the hood to equilibrate to room temperature, and then carefully vortexed to ensure thorough mixing of the contents. Only ten mice were dosed from each 1-mL HD vial, with the cap being replaced between animals to minimize evaporation of the methylene chloride. Five control animals were dosed first, followed by 10 pre-treatment or treatment animals, and then subsequent treatment groups of 10 animals each, followed by the last five control animals at the end of the study. A 5 μ L HD dose confirmation sample was placed into a 5-mL volumetric flask with hexane, and samples

also were taken after dosing with the first, second, third, etc., vials of HD. MREF chemistry staff analyzed these samples to confirm the expected concentration of HD. The chemistry staff documented dose confirmation analyses.

2.6 Biosampling

Approximately 24 hr after dosing, or at the assigned TAS harvest time, animals were euthanatized within the fume hood by placing them one at a time into a small glass desiccator which had been saturated with fumes from halothane poured onto a gauze pad. Once the animal had stopped breathing (approximately 2-3 min), the mouse was taken from the container and placed on a cutting board. The hair at the base of the ear was brushed back towards the body of the animal to aid in minimizing the adherence of loose hair to biopsy specimens. One or two scissors cuts were made at the base of the ear to remove each ear and the ear was laid, inner (anterior) surface upward, on the dissecting surface.

Timing from the point of euthanasia was important. One technician was responsible for euthanasia, another took biopsy punches, and a third measured weights. This was an integrated operation and the individual performing euthanasia adjusted the speed of this operation so specimens were not waiting to be weighed. An 8-mm disposable biopsy punch (Miltex Instrument Co., Inc., Lake Success, NY) was used to remove tissue from both ears. Care was taken to avoid twisting motion during the biopsy as twisting may introduce artifact into the histopathologic measures being made. The biopsy punches were placed into pre-labeled and tared (cap included in tare weight), 1.8-mL polypropylene, screw-cap vials color-coded for left and right ears. The cap was placed on the vial and the vial with its contents removed from the hood air space. A Mettler analytical balance was located immediately outside the dosing hood. The weight of the vial with tissue specimen and cap was recorded and then approximately 1.5 mL of 10 percent neutral buffered formalin was added immediately from a dispenser (vial was reopened in air space of hood system and formalin was added).

All specimens were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E) for evaluation. The study pathologist at Battelle's King Avenue facility evaluated these specimens for the presence or absence of histopathologic markers.

2.7 Study Endpoints

Inflammatory responses due to HD exposure are associated with an increase in cellular interstitial fluid. The increased interstitial fluid results in an increased tissue weight. The weights of the 8-mm tissue punches were taken to obtain an objective measure of tissue edema. The vial containing each sample was weighed and the tissue weight obtained by subtracting the tare weight of the vial and cap.

In certain laboratory species, HD is known to produce histopathologic changes similar to those observed in human tissue. These alterations include epidermal-dermal separation (limited separation in animal models - microblister formation), epidermal necrosis, follicular necrosis, intracellular edema, and pustular epidermitis. When a histopathologic marker was present within the lesion, a degree of severity score was assigned. The degree of severity ranged in value from "1" through "4". The scores for the pathologic changes indicate the following: "1" - involves <5 percent of the section; "2" - involves 10 to 40 percent of the section; "3" - involves 50 to 80 percent of the section; and "4" - involves >90 percent of the section. A score of 0 indicated no marker was present.

Microblister. A visible (at the light microscopy level) separation and loss of attachment of the epidermal basal cell layer with the underlying structures (i.e., basement membrane; not visible with routine staining). Must represent the loss, or dissolution, of at least 3 adjacent basal epithelial cells. Ulcerated areas will not be included in the microblister score. Frequently, within this newly created space, there will be cellular debris, neutrophils, and macrophages (i.e., a micropustule).

Epidermal Necrosis. Primarily refers to the nuclear morphology of the epidermal cells; includes condensation and shrinkage (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the most affected by HD. Microblisters and ulcerated areas are included in the necrosis scores. Sections containing scattered individual necrotic/dyskeratotic cells will be counted only if those cells appear in "clusters".

Contralateral Epidermal Necrosis. The definition and criteria are essentially the same as for epidermal necrosis. The inside part of the ear is exposed to HD; contralateral epidermal necrosis refers to the pathology occurring in the epidermal cells which comprise the outer part of the ear, beyond the cartilage.

Contralateral Microblister. The definition and criteria are essentially the same as for microblistering. Contralateral microblister formation may occur on the side of the ear not directly exposed to HD.

These histopathology criteria were selected after a meeting between the pathologists at Battelle and at USAMRICD in March 1997. Dr. Allen W. Singer read and scored the tissue slides from January 1997 through August 1998, at which time Dr. John D. Toft, II was trained to read the task slides by Dr. Singer. Both pathologists, to verify that both individuals were assigning histopathology scores similarly, read several sets of slides.

After conducting this task at the MREF for approximately two years, in order to accomplish a more efficient and timely evaluation of the histopathology scoring results, it was decided that only the right ears (all HD-exposed ears from HD-control and all treatment groups) would be evaluated for the presence or absence of histopathologic markers. Control ears (i.e., ears not exposed to HD and/or to compound) would not be evaluated routinely for histopathology and would be considered to be without pathology (i.e., scored 0). If there appeared to be an effect of a compound on the Relative Ear Weight (discussed in the next section), or histopathology results for the right ear specimen showed a statistically significant difference, then the corresponding left ear would be evaluated and a score assigned. This protocol change eliminated the time required for evaluating left ears except in cases where candidate compound activity was noted.

2.8 Statistical Analysis

The harvested mouse ear tissue biopsy wet-weight taken immediately after euthanasia was used to determine an index of edema, which was used as the primary quantitative response to tissue injury. This was called the relative ear weight (REW). REW was defined as the weight in milligrams of the HD-exposed ear (right ear) minus the weight of the control ear (left ear), divided by the weight of the control ear, and multiplied by 100. This parameter was determined for each animal. The mean percent REW of each candidate treatment group was statistically compared to that of the control group run on each day of testing to determine if the mean weight of HD-exposed ears of treated animals was statistically less than that for non-treated animals.

Based on the evaluation of HD dose-response and model validation data, evaluations were sometimes performed on transformed data. Statistical comparisons were performed using either an ANOVA or a one-sided, two-sample Student t-test, or analyses were conducted using analogous nonparametric methods.

Draft REW data were sent by facsimile to the sponsor within a day or two of testing so that the client was aware of compound testing results almost immediately. It usually took several weeks to process tissues and to allow the study pathologist time to read and score the slides. Once the Study Director received the histopathology scores, they were entered into a spreadsheet and sent electronically to the study statisticians for analyses.

For each histopathology endpoint, a one-sided Fisher's exact test was conducted to compare incidence of histopathologic endpoints between treated and control animals. Severity scores of histopathologic endpoints were summarized in tables. Categorical and/or nonparametric methods were used to statistically analyze the severity scores. All analyses were performed in SAS (Ver. 6.12). A summary table showing the results of the statistical comparisons was delivered in an Excel spreadsheet, along with the original data. Footnotes were used to note statistically significant results. All statistical hypothesis tests were conducted at the 0.05 level of significance.

Routinely, the left (control) ears were assigned an incidence and severity score of zero by the Study Director, to indicate the absence of any histopathologic markers (see protocol amendment 5). If, however, the initial REW indicated efficacy (i.e., the mean REW of a treatment group was much lower than that of the control group), then the study pathologist was asked to read and score the left ears for all control animals and for all animals in the suspected efficacious treatment groups. Once the data had been analyzed by the study statisticians, and if there appeared to be a positive effect of a compound on the REW, or the histopathology results for the right ear specimen showed a statistically significant difference and the compound passed the screen, then the Study Director notified the study pathologist to read and score the left ear of all control and all efficacious treatment group animals. If any of the left ears were scored other than zero by the pathologist, then the data is corrected on the spreadsheet and resubmitted to the study statisticians for re-analysis.

Within a week or so, the statistics were completed and sent again to the Study Director. Data were added (solubility data, study book reference number, amount of compound used, etc.) and reviewed on the final TAS. These were then forwarded (electronically) to an Army mailbox at USAMRICD, where the data were compiled and stored. From the time a compound was assigned to the MREF for testing via a TAS, final data were sent back to USAMRICD usually within 3-4 months.

3.0 RESULTS

The number of candidate compounds tested during the 33-month operation of Task 95-43 (January 1997 through October 6, 1999) was 334 compounds.

A candidate antivesicant drug was considered to have a significant effect when at least one tissue damage endpoint (i.e., relative ear weight, microblister formation, necrosis) provided a statistically significant reduction from its control. A table of the 73 compounds that passed the screen is included as Appendix B.

4.0 DISCUSSION

The majority of tests conducted at the MREF were initial screening studies designated as module AV300 of the USAMRICD Drug Assessment Branch antivesicant drug screening evaluations. This module was identified as the mouse ear pre-exposure test that utilized a single topical application of a candidate compound prior to HD challenge with a 24-hour tissue harvest time. Performing these studies at the MREF allowed the staff of USAMRICD to perform follow-up evaluations on promising candidate compounds without the need to utilize a majority of their resources to screen for efficacious compounds.

Candidate antivesicant drug testing was divided into six basic groups of drugs. Pretreatment times for administration of the drugs was established using known metabolic and pharmacokinetic data for each group of drugs. These groups and times of administration were:

Anti-inflammatories	-15 min
Anti-proteases	-60 min
PADPRP (poly-ADP-ribose polymerase) inhibitors	-30 min
Antioxidants and scavengers	-15 min
Cell cycle regulators	-60 min

Of the 73 passing compounds listed in Appendix B:

- 23 were anti-inflammatories
- 21 were protease inhibitors
- 15 were PADPRP inhibitors
- 12 were HD scavengers
- 2 were other classes of compounds

It is interesting to note that of these passing compounds, 29 were soluble in ethanol, 16 in acetone, 16 in DMSO, and one in methanol, and two were applied as ointments. Evidently, passing was not correlated with a specific solvent. No compounds passed only because of a reduction in the incidence of epidermal necrosis. Five compounds that passed only due to a reduction in microvesication warrant a more detailed inspection of other criteria, and therefore do not automatically advance to further modules -- this is because these compounds showed increases, not decreases, in other criteria measured. In some cases, microvesication is not measurable because the severity of damage, characterized by the fusion of the epidermal-dermal junction (known as liquefaction necrosis), results in no microvesication score.

Several compounds passing the initial screen were assigned testing as a 10-min post-treatment (AV330), with tissues harvested at 24 hr post HD exposure. Results of these advanced testing modules (see Table 2 of Appendix B) show that these compounds were efficacious as post-treatments. This is significant since, historically, it is documented that HD injury is not prevented unless intervention is initiated within 2 min of exposure. USAMRICD also documented the decreased effects of HD-induced tissue damage when these compounds were applied as a 10 min post-treatment after HD-exposure with tissues harvested at 24 hr.

A few compounds passing the initial screen also were assigned testing as a pre-treatment with tissue harvest at 48 hr (AV310) or 72 hr (AV320) after HD exposure. These compounds (see Table 2 of Appendix B) were shown to provide protection after 48 and even 72 hr when the compounds were administered in a single topical, pre-treatment dose.

Several visits by USAMRICD personnel throughout the course of this task provided opportunities for discussing and "fine-tuning" many technical procedures associated with this task. Histopathology criteria were altered following a meeting between the pathologists of Battelle and USAMRICD in March 1997 to better evaluate the severity and incidence of HD-induced tissue damage to the mouse ear in a similar and consistent manner. Statistics staff from both Battelle and USAMRICD also met on several occasions to develop the appropriate analysis parameters for this task and to create user-friendly programs and spreadsheets for electronic manipulation and transfer of the experimental data.

In April 1998, the scope of work of this task was increased to test 240 more compounds, in addition to the 72 compounds already tested to this point. In December 1998, histopathology readings of all left ear slides was eliminated, except in cases where candidate compound activity was noted, to save time in returning completed data packages to USAMRICD.

Procedures for dosing HD dilutions in methylene chloride, pre- and post-treatment applications, appropriate anesthetic regimens, animal manipulations, biopsy sampling techniques, histopathology scoring evaluations, meaningful statistical analyses and electronic reporting procedures were mastered by the task team members. To further test promising candidate antivesicant compounds, alternative routes of administration, multiple drug doses and/or longer observation times may be utilized in future work assignments to the MREF.

5.0 RECORD ARCHIVES

Records pertaining to the conduct of this task are contained in files maintained at the MREF. Many electronic files are also maintained. Continuation of this task under another MREF contract will necessitate keeping previous records and files open and available until such time as no further work is assigned. Various study records and files will be archived by USAMRICD or at Battelle at the close of the project. Task results are compiled electronically,

and therefore consist mainly of copies of previously reported results. Tissue specimens, blocks, and slides, will be discarded after acceptance of the Final Report at the close of the project.

6.0 ACKNOWLEDGEMENTS

The names, titles (role in the task), and highest degree(s) of the principal contributors to this study are presented in the following list.

<u>Name</u>	<u>Title</u>	<u>Degree</u>
James A. Blank	Study Director (1996-1997)	Ph.D.
Robyn C. Kiser	Study Director (1997-present)	B.S., RLATG
Carl T. Olson	Principal Investigator	D.V.M., Ph.D.
D. Marie Moore	Study Supervisor	LATG
Allen W. Singer	Study Pathologist	D.V.M.
John D. Toft, II	Study Pathologist	D.V.M.
Nancy A. Niemuth	Study Statistician	M.S.
Shawn M. Shumaker	Statistician	M.A.S.
James E. Estep	MREF Manager	D.V.M., Ph.D.


There are a number of people who made performance of this task possible. The authors gratefully acknowledge their valuable assistance. Among the many are: Tracy Peace, Frances Reid, and Robert Hunt for veterinary assistance; Theodore Miller, Janet Ricks, Jonathon Kohne, Harold Nitz, Heather McConeghy, and Charity Tucker for chemistry assistance, environmental monitoring, and dose analysis; Kandy Audet, Linda Baker, Michelle Clagett, Gary Finchum, Amy Forrest, William Hart, Robert Jarvis, Pamela Kinney, James Mann, Jennifer Quick, Reba Ryan, Mindy Stonerock, Jean Truxall, Michelle Tussing, and Jack Waugh for outstanding performance of technical tasks; Elisha Morrison and Jessica Evans for quality assurance oversight; Tami Kay for business and contract administration oversight; Meg Stalter for assistance in ordering animals; Kerrie Copas for statistical analysis support; William Ritter for test article identification assistance; David Stitcher for safety oversight; and Charlotte Hirst for secretarial support and report preparation.

APPENDIX A


Protocol 116

Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and
Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard
Exposure at the Medical Research and Evaluation Facility

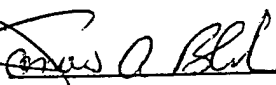
Study Performed by Battelle Memorial Institute,
505 King Avenue, Columbus, Ohio 43201-2693


John B. Johnson, D.V.M., M.S.
Manager, Medical Research and Evaluation Facility
and Co-Principal Investigator


6/20/96
Date


Carl T. Olson, D.V.M., Ph.D.
Co-Principal Investigator

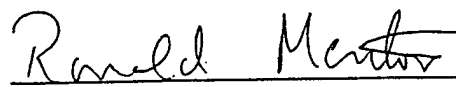
6/20/96
Date


James A. Blank, Ph.D.
Study Director

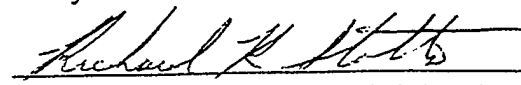
19 June 1996
Date


Tracy A. Peace, D.V.M.
Attending/Consulting Veterinarian

062696
Date


Ronald G. Menton, Ph.D.
Study Statistician

6/25/96
Date


LTC Richard R. Stotts, D.V.M., Ph.D., COR,
U.S. Army Medical Research Institute of Chemical
Defense (USAMRICD)

21 JUN 96
Date

Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and
Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard
Exposure at the Medical Research and Evaluation Facility

PRINCIPAL INVESTIGATOR: Carl T. Olson, D.V.M., Ph.D.

- I. NON-TECHNICAL SYNOPSIS: Sulfur mustard (HD) vapor on skin produces no immediate reaction, but penetrates the skin rapidly. With time, an erythema (reddening of skin) of increasing severity is observed, followed by fluid accumulation at the site of contact. The mouse ear model is a model for assessment of inflammatory injury by HD with the physiological basis being fluid accumulation due to capillary damage. The model has been used by investigators at USAMRICD to evaluate candidate pre- and post-treatments for percutaneous HD exposure. This model is being transitioned and will be utilized at Battelle's Medical Research and Evaluation Facility (MREF) for the evaluation of additional candidate pre- and post-treatments.
- II. BACKGROUND: The effects of HD exposure range from mild erythema to vesication and even complete destruction of tissue. Although HD penetrates skin rapidly, the macroscopic skin changes are delayed for hours. HD exposure is accompanied by physiological and biochemical processes which result in local cellular changes along with increased capillary permeability and edema. The increased capillary permeability or the blood vessel damage caused by vesicants forms the basis of the mouse ear model of Brinkley et al. (1989) who showed that the accumulation of extravasated I^{125} labeled albumin was HD dose dependent. Others have shown that the mouse ear-swelling model is a more quantifiable and objective model than models based on subjective evaluation of skin changes for investigative phototoxicity testing (Gerberick and Ryan, 1989). The anti-inflammatory activity of corticoids has been evaluated in rat ears in which the increase in weight gain (edema) in a croton oil treated ear is compared with the non-treated ear (Tonelli et al., 1966).
- III. OBJECTIVE: The mouse ear model was established at USAMRICD by Brinkley et al. (1989) to assess vesicant-induced skin damage and was extended further by Casillas et al. (1996) to evaluate pre- and post-treatment compounds against HD exposure. This protocol is to transition the model from USAMRICD to the MREF and to implement the

model for assessing pre- and post-treatment compounds for the treatment of vesicant injury.

IV. MILITARY RELEVANCE: The use or threat of HD use in military conflicts has stressed the need for the development of antivesicant compounds, and the characterization of a suitable animal model for screening potential prophylactic and therapeutic compounds to prevent or treat vesicant agent injury. The need for such a model is expressed in the requirements of Joint Service Agreement (JSA) S-A-301 (Prophylactic Drugs), S-A-302 (Antidotes), S-A-303 (CW and BW Therapeutic Drugs), and C-A-303 (Pretreatment Models), and in STO A objectives.

V. MATERIALS AND METHODS:

A. Experimental Design:

1. Testing Category: Testing category, or how a particular candidate compound is to be screened, is assigned by USAMRICD and provided in a Test Assignment Sheet (TAS).
2. Model Transition to the MREF:
 - a. HD Dose Response - This study is to establish a HD challenge dose that produces a degree of edema similar to that used by investigators at USAMRICD for candidate compound evaluations. Mice will be exposed to three HD doses and edema will be measured at 24 hr following exposure. Each dose group will consist of 10 animals and exposures will be performed as described in a later section. Measures of edema and histopathology will be performed on these samples.
 - b. Model Validation - Successful transition of the model to the MREF will be determined by qualitatively duplicating the edema and histopathologic results for four compounds that have already been evaluated at USAMRICD. The compounds will be evaluated as described in the compound evaluation section. Measures of edema and histopathology will be made in this section.

3. Assessment of Topical Pre- and Post-treatments:

- a. If not already determined or already formulated, the solubility of candidate compounds in various solvents are determined according to MREF Protocol 76. Solvents to be evaluated include ethanol, acetone, and saline solution, or others as specified by the sponsor.
- b. Each treatment group will consist of 10 animals. A treatment group which has not received a pre- or post-treatment but has been exposed to HD will be included in each experimental setup to serve as the control.
- c. Candidate compounds (nominally 1 mg) are applied to the inner, central part of the left and right ears of animals. The dose may be delivered as a single, approximately 5 μ L dose, or may be administered as repeated applications to the same skin surface region. For pre- and post-treatments, the time interval prior to or following HD exposure for compound application will be provided by the sponsor.
- d. The animals are weighed and anesthetized prior to HD application. Approximately 5 μ L of methylene chloride is applied to the left ear and approximately 5 μ L of HD diluted in methylene chloride is applied to the right ear. The HD concentration will be determined during the model transition phase. For HD application, the animals are positioned in addition to being anesthetized (e.g., limbs taped to a surface). HD exposures are performed on a group of animals receiving experimental treatment as well as on a group not treated. The ears are not decontaminated following HD exposure.
- e. Following exposure the animals are placed in polycarbonate cages and retained within the fume hood system with food and water for approximately 24 hr.
- f. Approximately 24 hr following HD exposure, the animals are euthanatized by carbon dioxide or halothane overdose. The ears are cut from the animal and 8 mm punches of the exposed tissues are taken. The 8 mm punches are weighed, then either placed in a neutral buffered formalin solution for histopathology or snap-frozen in liquid nitrogen and stored at approximately - 70 degrees C.

- g. Edema and histopathologic changes are determined and statistically evaluated as described in a later section.

B. Laboratory Animals and Justification

1. Non-animal Alternatives Considered - Non-animal alternatives are not feasible as the responses of interest require an integrated physiological system. The edemic response being examined requires interaction of the skin with the systemic circulation and with other organs such as the liver.
2. Animal Model and Species Justification - The mouse has been used for years in assessing dermal irritation, and has been shown to exhibit an edemic response following HD exposure (Brinkley et al., 1980; Casillas et al., 1996). Few laboratory animal species are known to microvesiculate following HD exposure. The mouse ear has been shown recently to microvesiculate following percutaneous HD exposure (Casillas et al., 1996).
3. Model System:
 - a. Genus & Species: *Mus musculus*
 - b. Strain/Stock: Crl:CD-1 (ICR) BR
 - c. Source/Vendor: Charles River Laboratories
 - d. Age - Mice will be at least four weeks old upon arrival.
 - e. Weight - Mice weighing in the approximate range of 25 to 35 g are used on study.
 - f. Sex - Male
4. Other - Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study.
5. Number of Mice Required:

- a. Transition Studies - 110 mice
- b. Compound Evaluation - 20 mice per evaluation
- c. Fourteen extra mice for transition studies and four extra mice per compound evaluation are requested to cover technical problems. If additional animals are needed due to technical problems, unavoidable circumstances, or enhanced work scope, the IACUC procedures for requesting approval for additional animals will be followed.

6. Refinement, Reduction, Replacement

- a. Refinement - Animals will be anesthetized during the period of HD exposure.
- b. Reduction - Statistical calculations based upon known variability have been made to minimize the number of animals utilized per treatment group. Candidate compound evaluations will be evaluated in batch format, whenever possible, to minimize animal utilization through the use of a single set of concurrent controls.
- c. Replacement - *In vitro* models cannot simulate the complex physiologically integrated toxic reaction induced by HD, nor account for all aspects of protection afforded by the candidate compounds. Once additional information regarding the mechanism of HD-induced toxicity is available, it may be possible to develop *in vitro* models to perform limited evaluations of candidate compounds.

C. Technical Methods

1. Pain:

USDA (Form 18-3) Pain Category

- a. No Pain: Category C; 0 mice
- b. Alleviated Pain: Category D; 110 mice for the validation study;
20 mice per compound evaluated
- c. Unalleviated Pain or Distress: Category E; 0 animals
- d. Pain Alleviation

- (1) Anesthesia/Analgesia/Tranquilization: Animals are anesthetized during the HD exposure period using appropriate doses of ketamine and xylazine administered intramuscularly (i.m.).

Previous experience has indicated that pain or distress after HD exposure and decontamination does not exist. However, should evidence of pain or distress be noted during the study (e.g., decreased activity, anorexia, or self mutilation), pain will be alleviated with buprenorphine at an approximate dose of 2.5 mg/kg administered subcutaneously (s.c.) or intraperitoneally (i.p.). External stimuli and manipulation are minimized to decrease any associated anxiety.

- (2) Paralytics: Not applicable

e. Alternatives to Painful Procedures

- (1) Source(s) Searched: Medlars/Grateful Med
- (2) Date of Search: 1980 through 1996
- (3) Key Words of Search: sulfur mustard and pain.
- (4) Results of Search: No records of the key word combinations shown above were found.

- f. Painful Procedure Justification - No pain or discomfort to the animals is expected to result from cutaneous administration of either HD or the drugs used in this study.

2. Prolonged Restraint: Not applicable
3. Surgery: Not applicable
4. Dilution of HD: HD is diluted in methylene chloride following procedures outlined in MREF Standard Operating Procedure (SOP) I-002.

5. Animal Manipulations:

- a. Animal Identification - Animals are identified by tail tattoo, cage cards, or other appropriate means.
- b. Injections - Mice are injected with anesthetic/analgesia either i.p., s.c., or i.m., using a 23 - 25 g needle affixed to a 1-mL tuberculin syringe.
- c. Behavioral Studies: not applicable
- d. Animal Preparation - On the day of study, the animals are weighed to the nearest 0.5 gm using a calibrated balance.
- e. HD Exposure - HD is applied to anesthetized animals following the procedures outlined in MREF SOP II-009. Approximately 5 μ L of the diluent, methylene chloride, is applied to the inner surface of the left ear. To the same animal, approximately 5 μ L of diluted HD is applied on the inner surface of the right ear. These doses are applied as point droplets. Once the methylene chloride has evaporated, the animal is placed into its cage and maintained within the fume hood system. The dosed area is not decontaminated following exposure. The cap to the diluted HD solution should be replaced after each dose sample has been extracted to minimize evaporation of the methylene chloride.
- f. Dose Confirmation Samples - Since HD will be contained in a volatile solvent (i.e., methylene chloride), dose confirmation samples may be taken prior to and after completion of HD dosing to verify that significant evaporation of methylene chloride with a resulting increase in HD concentration has not occurred. For dose confirmation, the dosing solution is diluted below chemical surety limits following SOP MREF I-002. The diluted samples are placed into gas chromatograph vials, capped, and transferred to chemistry following SOP MREF I-003. The samples are analyzed for HD content following SOP MREF III-002.
- g. Biosamples - Animals are euthanatized within the fume hood. Both ears of each animal are removed using a pair of sharp scissors, and the ear is laid, inner surface upward, on a dissecting surface. An 8-mm biopsy

punch is used to remove the HD-exposed tissue. The tissue is placed into a tared vial and the weight is obtained. The tared vial has not been contained within the fume hood, but is held inside the airspace of the hood by an individual wearing clean double surgical gloves. The vial cap is secured and the vial removed from the hood airspace and the weight of the vial determined. The sample is then either placed in neutral buffered formalin solution or snap-frozen in liquid nitrogen (SOP GEN VII-003) and stored at approximately -70 C. The vial containing the sample is opened inside a biological safety cabinet or a fume hood by an individual wearing a laboratory coat and double surgical gloves to submerge the sample in neutral buffered formalin. Samples stored at -70 C are appropriately labeled to indicate contents and date of exposure to HD.

6. Adjuvants - not applicable
7. Study Endpoints:
 - a. Wet Weight - Inflammatory responses are associated with an increase in interstitial fluid. The increased interstitial fluid should result in an increased tissue weight. The weight of the 8-mm tissue punches are taken to obtain an objective measure of tissue edema. The vial containing sample is weighed and the tissue weight is obtained by subtracting the tared weight of the vial.
 - b. Histopathology - In certain laboratory animals species, HD is known to produce histopathologic changes similar to that observed with human tissue. These alterations include epidermal-dermal separation (limited separation in animal models - microblister formation), epidermal necrosis, follicular necrosis, intracellular edema, and pustular epidermitis.

All specimens are fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E) for evaluation. Neutral buffered formalin specimens will be evaluated for the presence or absence of histopathologic markers. When a histopathologic marker is present within the lesion, a degree of severity score will be assigned. The degree of severity ranges in value from "1"

through "4". A "1" indicates that the pathologic change is negligible and only occurs in one or two discrete foci. A "4" indicates that the lesion is severe and diffuse and could get no worse. Both "2" and "3" span the intermediate zone and take into account both amount and distribution of damage. The histopathologic markers have the following definitions:

- (1) Intracellular edema (ballooning degeneration, hydropic degeneration, vacuolar degeneration) of epidermis. Characterized by increased size, cytoplasmic pallor, and nuclear displacement to the periphery of affected cells; refers to all layers of the epidermis.
 - (2) Epidermal necrosis. Primarily refers to the nuclear morphology of the epidermal cells; includes condensation and shrinkage (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the cells most affected by HD.
 - (3) Pustular epidermitis. The presence within the epidermal layer of neutrophils which are not present under normal conditions and without the appropriate stimuli (inflammatory mediator release).
 - (4) Microblister. A visible (at the light microscopy level) separation and loss of attachment of the epidermal basal cell layer with the underlying structures (i.e., basement membrane; not visible with routine staining). Must represent the loss, or dissolution, of at least two adjacent basal cells. Frequently, within this newly created space there will be cellular debris, neutrophils, and macrophages (i.e., a micropustule).
 - (5) Follicular necrosis. Refers to the necrosis of the basal cell layer and other epidermal layers which are found invaginating into the dermis and lining the hair follicle.
8. Euthanasia: Mice will be sacrificed by halothane or carbon dioxide overdose, or other current AVMA panel approved methods .

D. Veterinary Care:

1. Husbandry Considerations: Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. Animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health (NIH) on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended.

Accreditation - On January 31, 1978, Battelle's Columbus Operation received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

- a. Mice - Animals may be group housed or housed individually in polycarbonate cages. Food and water are provided ad libitum except on the morning of HD exposure. Following HD exposure, the animals are group housed unless individual housing is needed. The animals are housed in polycarbonate cages held in a chemical fume hood system for approximately 24 hr with access to food and water. As needed, the animal cages may be held on a warm-water perfused heating pad.
- b. Animals are quarantined for a minimum of 5 days.
- c. Fluorescent lighting is used with a 12-hr light cycle per day, except when animals are maintained for approximately 24 hr following exposure in a chemical fume hood.
- d. Temperature - Maintained at 64 to 79 degrees F. At least 90 percent of the twice-daily readings will fall within the specified range.

- e. Relative humidity - Maintained at 40-70 percent. At least 90 percent of the twice-daily readings will fall within the specified range.
 - f. Diet - Purina Rodent Chow is available ad libitum except immediately prior to study.
 - g. Water - Water is supplied from Battelle's West Jefferson water system and is available ad libitum except immediately prior to study. No contaminants which would affect the results of the study are known to be present in the water.
 - h. Special Husbandry Considerations: Not applicable
2. Attending Veterinary Care
- a. Animals are examined upon receipt.
 - b. Animals are examined at least once a day by trained technical personnel. If any problems are observed, a staff veterinarian is notified.
 - c. On the day of exposure and periodically throughout the post-exposure time period, animals are observed by trained personnel. If an animal becomes moribund, it will be euthanatized.
3. Enrichment Strategy: Polyvinyl chloride tubing (approximate internal diameter 4 in) cut in half lengthwise or plastic orange balls the size of golf balls may be inserted in the cages.
- E. Data Analysis: All statistical hypothesis tests are conducted at the 0.05 level.
- 1. HD dose-response studies: Relative ear weight (REW) is the primary response variable where REW is defined to be the ear weight of the HD-exposed ear divided by the weight of the ear not exposed to HD. For each group of animals, a one-sample student t-test is conducted to determine if the mean ear weight of HD exposed ears is statistically different than that of ears not exposed to HD. This will be accomplished by statistically comparing the mean REW of each group of animals to one. In addition, mean REW of the three HD dosed groups are statistically compared using an analysis of variance

(ANOVA) model. Multiple comparisons among the three groups of animals are performed using Tukey's method. The assumption of approximate normality for the distribution of REWs is assessed visually. If this assumption is grossly violated, then either a transformation may be applied to the data prior to carrying out the tests, or the analysis may be conducted using nonparametric or categorical methods.

If data from tests conducted at USAMRICD are provided to Battelle, then a statistical test is conducted for each HD dose group to compare the mean REW between experiments conducted at USAMRICD and Battelle.

2. Model Transition: REW and incidence and severity of histopathology are statistically compared among the four compounds at the MREF to determine if statistical differences among the four compounds are comparable to those obtained at USAMRICD. An ANOVA is carried out to statistically compare the mean REW of the four compounds and multiple comparisons among the four compounds are performed using Tukey's method. The assumption of approximate normality for the distribution of REWs is assessed visually. If this assumption is grossly violated then either 1) a transformation may be applied to the data prior to carrying out the ANOVA, or 2) the analysis may be conducted using nonparametric or categorical methods.

Chi-square tests are conducted to compare incidence of microblisters among the four compounds. Tables of frequency counts are used to present results of severity scores. Categorical and/or nonparametric methods may be used to statistically analyze the severity scores.

Statistical differences obtained at Battelle are qualitatively compared to those obtained at USAMRICD. If experimental data from tests conducted at USAMRICD are provided to the Battelle, then a two-way ANOVA may be performed to compare the mean REWs between experiments conducted at USAMRICD and Battelle.

3. Assessment of Topical Pre- and Post-Treatments: Mean REW of each candidate treatment is statistically compared to that of the control group to determine if the mean weight of HD-exposed ears for treated animals is statistically less than that for non-treated animals. Based on the evaluation of HD dose-response and model validation data, evaluations may be performed on

transformed data. Statistical comparisons are performed using either an ANOVA test or a one-sided, two-sample Student t-test. Analysis may be conducted using analogous nonparametric methods.

For each histopathologic endpoint, a one-sided Fisher's exact test is conducted to compare incidence of histopathologic endpoints between treated and control animals. Severity scores of histopathologic endpoints are summarized in tables. Categorical and/or nonparametric methods may be used to statistically analyze the severity scores.

- F. Investigator and Technical Qualifications/Training: All technical staff members involved in the receipt, care, and use of animals are AALAS certified laboratory animal technicians or in training for certification. All training documentation is maintained in personnel training files. Dr. James Blank has four years of experience in the handling and use of rodent species for experimental purposes. Dr. Ronald Menton is a biostatistician with over 7 years experience in designing biostudies and applying designs to minimize and reduce animal usage. Dr. Carl Olson has his veterinary medicine degree and has had over 30 years of experience in working with rodent and large animal species. Dr. Tracy Peace has a veterinary medicine degree and is certified by the American College of Laboratory Animal Medicine.

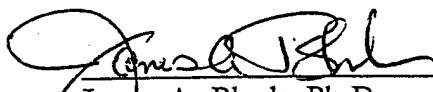
- VI. Biohazard/Safety: The chemicals and hazardous wastes used or generated in this protocol will be handled in accordance with all applicable state and federal guidelines, regulations and standing operating procedures to ensure that no significant adverse environmental effects occur. All HD Chemical Surety Materiel (CSM) work will be conducted in accordance with all applicable Facility Safety and Standard Operating Procedures. Standard monitoring procedures are in place to assure safety in conjunction with the use of HD.

- VII. Assurances: As Study Director on this protocol I acknowledge my responsibilities and provide assurances for the following:

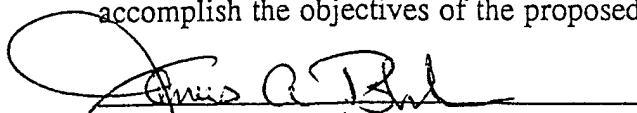
- A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a deviation is specifically approved by the IACUC.
- B. Duplication of Effort: These studies have been performed at USAMRICD. Some duplication of effort will be performed in the validation study to assess successful

transition of the procedure from USAMRICD to Battelle. The model will then be used to assess the efficacy of candidate compounds against HD-induced skin damage. Since the identity of these candidate compounds is unknown to the evaluator, it is assumed that this will not be a duplication of effort.

- C. Statistical Assurance: I assure that I have consulted with an individual who is qualified to evaluate the statistical design or strategy of this proposal, and that the minimum number of animals needed for scientific validity are used.
- D. Biohazard/Safety: I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, etc., in the preparation of this protocol.
- E. Training: I verify that the personnel performing the animal procedures/manipulations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused as a result of the procedures/manipulations.
- F. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R" which the DOD has embraced, namely, "Responsibility" for implementing animal use alternatives where feasible, and conducting humane and lawful research.


James A. Blank, Ph.D.

- G. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals that will be relieved with the use of anesthetics, analgesics, and/or tranquilizers. I have considered alternatives to such procedures; however, using the methods and sources described in the protocol, I have determined that alternative procedures are not available to accomplish the objectives of the proposed experiment.


James A. Blank, Ph.D.

IX. Enclosures:

- A. Literature Searches
- B. Pathology Addendum: not applicable

X. References:

Brinkley, F. B., M.M Mershon, S. Yaverbaum, B.F. Doxzon, and J.V. Wade. 1989. The Mouse Ear Model as an In Vivo Bioassay for the Assessment of Topical Mustard (HD) Injury, *In* Proceedings of the 1989 Medical Defense Bioscience Review, pp 595-602, U.S. Army Medical Research and Development Command. AD B139550

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Gerberick, G. F., and C.A. Ryan. 1989. A Predictive Mouse Ear-Swelling Model for Investigating Topical Phototoxicity. *Food Chem. Toxicol.* 27: 813-819.

Tonelli, G., L. Thibault, and I. Ringler. 1965. A Bio-assay for the Concomitant Assessment of the Antiphlogistic and Thymolytic Activities of Topically Applied Corticoids. *Endocrinology* 77: 625-634.

**Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and
Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard
Exposure at the Medical Research and Evaluation Facility**

Protocol Amendment No. 1

Change No. 1: Page 3, Section V.A.2.a.

Change from: Mice will be exposed to three HD doses and edema will be measured at 24 hr following exposure.

Change to: Mice will be exposed to three HD doses and edema will be measured at 24 hr following exposure. Additional experimental stages of exposure may be performed to more fully characterize the HD dose response relationship with respect to edema and histopathology as needed to demonstrate model transition from USAMRICD to the MREF.

Reason for Change: The first exposure day of animals to three doses of HD provided insufficient data to demonstrate model transition. An additional day to expose animals is needed. The protocol is amended to reflect this need as well as the possibility of additional exposure days to demonstrate transition.

Impact on Study: This change does not have an impact on the data integrity.

Change No. 2: Page 6, Section V.B.5.a.

Change from: Transition Studies - 110 mice

Change to: Transition Studies - 140 mice, minimally.

Reason for Change: Additional exposure days under the model transition phase are required to demonstrate transition from USAMRICD to the MREF. An additional 30 mice at a minimum will be used for this phase. At this time, the total number of animals to be used under this protocol will not be increased.

Impact on Study: This change does not have an impact on data integrity

Change No. 3: Page 6, Section V.C.1.b.

Change from: Alleviated Pain: Category D; 110 mice for the validation study; 20 mice per compound evaluated

Change to: Alleviated Pain: Category D; 110 mice, minimally for the validation study; 20 mice per compound evaluated

Reason for Change: Additional exposure days under the model transition phase are required to demonstrate transition from USAMRICD to the MREF. An additional 30 mice at a minimum will be used for this phase. At this time, the total number of animals to be used under this protocol will not be increased.

Impact on Study: This change does not have an impact on the data integrity.

Change No. 4: Page 7, Section V.C.1.d.(1)

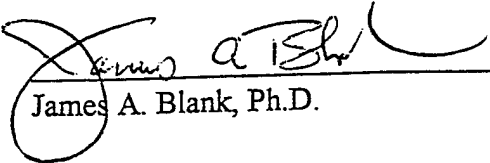
Change from: Mice are anesthetized with a combination of ketamine hydrochloride (6.1 mg/kg) and xylazine (1.1 mg/kg) administered intramuscularly (i.m.).

Change to: Mice are anesthetized with a combination of ketamine hydrochloride and xylazine administered via intramuscular (i.m.), intraperitoneal (i.p.), or subcutaneous (s.c.) route at dosages to be determined by veterinary staff.


Reason for Change: The route of exposure for initial anesthesia administration is the i.p. route. The dosages and routes required for anesthesia may change pending recommendation by veterinary staff. Dosages and routes of administration other than i.p. will be documented in the study file.

Impact on Study: This change does not have an impact on the data integrity.

Approved by:


James A. Blank, Ph.D.

21-MAR-97
Date


LTC Richard R. Stotts, D.V.M., Ph.D., COR

21 MAR 97
Date

Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard Exposure at the Medical Research and Evaluation Facility

Protocol Amendment No. 2

Change No. 1: Page 4, Section V.A.3.c.

Change from: Candidate compounds (nominally 1 mg) are applied to the inner, central part of the left and right ears of animals. The dose may be delivered as a single, approximately 5 uL dose, or may be administered as repeated applications to the same skin surface region. For pre- and post-treatments, the time interval prior to or following HD exposure for compound application will be provided by the sponsor.

Change to: Candidate compounds (nominally 1 mg) are applied to the inner, central part of the right ear of animals. The dose may be delivered as a single, approximately 5 - 10 uL dose, or may be administered as repeated applications to the same skin surface region to obtain the total required dose volume. For pre- and post-treatments, the time interval prior to or following HD exposure for compound application will be provided by the sponsor.

Change No. 2: Page 9, Section V.C.7.b.

Change from: Histopathology - In certain laboratory animals species, HD is known to produce histopathologic changes similar to that observed with human tissue. These alterations include epidermal-dermal separation (limited separation in animal models - microblister formation), epidermal necrosis, follicular necrosis, intracellular edema, and pustular epidermitis.

All specimens are fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E) for evaluation. Neutral buffered formalin specimens will be evaluated for the presence or absence of histopathologic markers. When a histopathologic marker is present within the lesion, a degree of severity score will be assigned. The degree of severity ranges in value from "1" through "4". A "1" indicates that the pathologic change is

negligible and only occurs in one or two discrete foci. A "4" indicates that the lesion is severe and diffuse and could get no worse. Both "2" and "3" span the intermediate zone and take into account both amount and distribution of damage. The histopathologic markers have the following definitions:

- (1) Intracellular edema (ballooning degeneration, hydropic degeneration, vacuolar degeneration) of epidermis. Characterized by increased size, cytoplasmic pallor, and nuclear displacement to the periphery of affected cells; refers to all layers of the epidermis.
- (2) Epidermal necrosis. Primarily refers to the nuclear morphology of the epidermal cells; includes condensation and shrinkage (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the cells most affected by HD.
- (3) Pustular epidermitis. The presence within the epidermal layer of neutrophils which are not present under normal conditions and without the appropriate stimuli (inflammatory mediator release).
- (4) Microblister. A visible (at the light microscopy level) separation and loss of attachment of the epidermal basal cell layer with the underlying structures (i.e., basement membrane; not visible with routine staining). Must represent the loss, or dissolution, of at least two adjacent basal cells. Frequently, within this newly created space there will be cellular debris, neutrophils, and macrophages (i.e., a micropustule).
- (5) Follicular necrosis. Refers to the necrosis of the basal cell layer and other epidermal layers which are found invaginating into the dermis and lining the hair follicle.

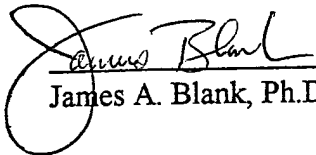
Change to: Histopathology - In certain laboratory animal species, HD is known to produce histopathologic changes similar to that observed with human tissue. These alterations include epidermal-dermal separation (limited separation in animal models-microblister formation), epidermal necrosis, follicular necrosis, intracellular edema, and pustular epidermitis.

All specimens are fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E) for evaluation. Neutral buffered formalin specimens will be evaluated for the presence or absence of histopathologic markers. When a histopathologic marker is present within the lesion, a degree of severity score will be assigned. The degree of severity ranges in value from "1" through "4". The scores for the pathologic changes indicate the following: "1" - involves <5 percent of the section; "2" - involves 10 to 40 percent of the section; "3" - involves 50 to 80 percent of the section; and "4" - involves >90 percent of the section.


- (1) Microblister. A visible (at the light microscopy level) separation and loss of attachment of the epidermal basal cell layer with the underlying structures (i.e., basement membrane; not visible with routine staining). Must represent the loss, or dissolution, of at least 3 adjacent basal epithelial cells. Ulcerated areas will not be included in the microblister score. Frequently, within this newly created space, there will be cellular debris, neutrophils, and macrophages (i.e., a micropustule).
- (2) Epidermal Necrosis. Primarily refers to the nuclear morphology of the epidermal cells; includes condensation and shrinkage (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis) of the nucleus. Basal cells are the most affected by HD. Microblisters and ulcerated areas are included in the necrosis scores. Sections containing scattered individual necrotic/dyskeratotic cells will be counted only if those cells appear in "clusters".
- (3) Contralateral Epidermal Necrosis. The definition and criteria are essentially the same as for epidermal necrosis. The inside part of the ear is exposed to HD, contralateral epidermal necrosis refers to necrosis occurring in the epidermal cells which comprise the outer part of the ear beyond the cartilage.

Reason for Change: Application of the pre- and post-treatment compounds to both ears may cause a systemic exposure when the evaluation is examining topical drug delivery to a specific site. Application to the ear being exposed to HD reduces the possibility of observing a systemic effect. The histopathology criteria were altered following a meeting between the pathologists at Battelle and USAMRICD. The modified criteria reflect the changes.

Approved by:


James A. Blank, Ph.D.

12 May - 97
Date


LTC Richard R. Stotts, D.V.M., Ph.D., COR

12 May 97
Date

**Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and
Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard
Exposure at the Medical Research and Evaluation Facility**

Protocol Amendment No. 3

Change No. 1: Page 4, Section V.A.3.e.

Add: For studies to assess extended protection afforded by a candidate pre- or post-treatment, animals may be housed in polycarbonate cages within the fume hood system for up to approximately 72 hrs following exposure. During this period, food and water will be provided and environmental conditions monitored.

Reason: The client desires to perform extended evaluatory studies with respect to time for those candidate pre- and post-treatment compounds affording protection at 24 hrs following exposure.

Change No. 2: Page 4, Section V.A.3.f.

Change from: Approximately 24 hr following HD exposure, the animals are euthanatized by carbon dioxide or halothane overdose. The ears are cut from the animal and 8 mm punches of the exposed tissue are taken. The 8 mm punches are weighed, then either placed in a neutral buffered formalin solution for histopathology or snap-frozen in liquid nitrogen and stored at approximately -70 degrees C.

Change to: At the indicated time following exposure (approximately 24, 48, or 72 hr), the animals are euthanatized by carbon dioxide or halothane overdose. The ears are cut from the animal and 8 mm punches of the exposed tissue are taken. The 8 mm punches are weighed, then either placed in a neutral buffered formalin solution for histopathology or snap-frozen in liquid nitrogen and stored at approximately -70 degrees C.

Reason: The time of euthanatization may be either 24, 48, or 72 hrs depending on whether the client requests an extended time evaluation study or not.

Change No. 3: Page 6, Section V.B.5.b.

Change from: Compound Evaluation -20 mice per evaluation

Change to: Compound Evaluation - up to 60 mice per evaluation

Reason: Candidate compound evaluations may consist of a single evaluation at 24 hr, consist of an extended time evaluation, or consist of a dose-response study. A single evaluation at 24 hr may utilize as many as 20 mice per compound, an extended time evaluation may utilize as many as 40 mice per compound, and a dose-response evaluation may utilize as many as 60 mice per compound.

Change No. 4: Page 18, Section V.C.1.b.

Change from: Alleviated Pain: Category D; 110 mice minimally for the validation study; 20 mice per compound evaluated.

Change to: Alleviated Pain: Category D; 110 mice minimally for the validation study; Up to 60 mice per compound evaluation.

Reason: The number of animals per evaluation may change pending upon whether a single 24 hr evaluation, extended time evaluation, or a candidate compound dose-response evaluation is performed.

Change No. 5: Page 11, Section V.D.1.a.

Change from: Mice - Animals may be group housed or housed individually in polycarbonate cages. Food and water are provided ad libitum except on the morning of HD exposure. Following HD exposure, the animals are group housed unless individual housing is needed. The animals are housed in polycarbonate cages held in a chemical fume hood system for approximately 24 hr with access to food and water. As needed, the animal cages may be held on a warm-water perfused heating pad.

Change to: Mice - Animals may be group housed or housed individually in polycarbonate cages. Food and water are provided ad libitum except on the morning of HD exposure. Following HD exposure, the animals are group housed unless individual housing is needed. The animals may be housed in polycarbonate cages held in a chemical fume hood system for up to

approximately 72 hr with access to food and water. As needed, the animal cages may be held on a warm-water perfused heating pad.

Reason: The length of housing in the chemical fume hood system may be up to approximately 72 hr for extended time evaluation studies.

Change No. 6: Page 11, Section V.D.1.c.

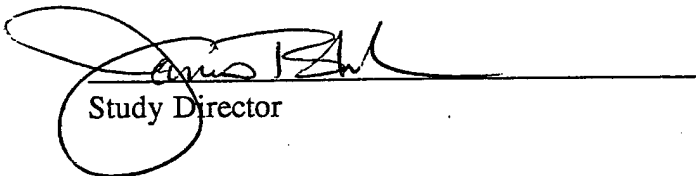
Add: When animals are maintained in a chemical fume hood system for extended time evaluation studies, the fluorescent lighting used will approximate a 12-hr light cycle per day.

Reason: The light cycle for extended periods of animal holding within the fume hood system will approximate 12 hr light cycles to maintain as normal hold period as is practical.

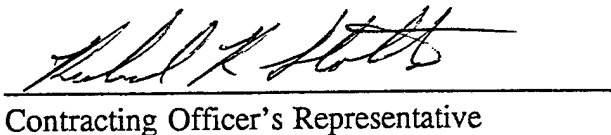
Change No. 7 Page 1

Change from: James Blank, Study Director

Change to: Robyn C. Kiser, Study Director


Study Director

8-Aug-97
Date


Contracting Officer's Representative

27 Aug 97
Date

Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and
Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard
Exposure at the Medical Research and Evaluation Facility

Protocol Amendment No. 4

Effective date: April 1, 1998


Addition No. 1: Page 6, Section V.B.5.d.

d. The client has requested that the scope of work of this task be extended to test approximately 240 additional compounds. Extending this task until January 1999, and scheduling the testing of 6 compounds per week, would require ordering 3,600 additional animals to utilize 3,200 mice. By evaluating three compounds per study day (10 mice per compound) in addition to 10 control animals, 40 mice plus 10% extra (4) are necessary to run each experiment. Extra animals are needed to make the 25-35 g weight range and have normal ears available for tissue sampling. Therefore, 88 mice per week are needed for 40 weeks. Charles River Labs, Portage, MI has been supplying 90 mice per week when 88 are requested. This extended scope of work assumes that the candidate compounds are available and ready for testing, 3 at a time, and that resources are available to conduct this task twice a week.


Reason for addition: The client has requested that an additional 240 candidate compounds be tested using this protocol.

Impact on study: None.

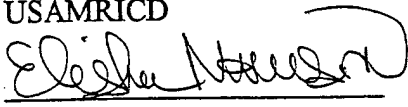
Approved:


Robyn C. Kiser, B.S., RLATG
Study Director

3-24-98
Date


LTC Richard R. Stotts, COR
USAMRICD

10 April 98
Date


Elisha N. Morrison, M.S.
Quality Assurance Specialist

4/13/98
Date

Transition and Use of the Mouse Ear Model for Evaluating Candidate Pre- and
Post-Treatments for Efficacy Against Percutaneous Sulfur Mustard
Exposure at the Medical Research and Evaluation Facility

Protocol Amendment No. 5

Effective date: November 24, 1998

Change No. 1: Page 1

Change from: John B. Johnson, D.V.M., M.S., Manager, MREF and Co-Principal
Investigator

Change to: James E. Estep, D.V.M., Ph.D., Manager, MREF

Change from: Carl T. Olson, D.V.M., Ph.D., Co-Principal Investigator

Change to: Carl T. Olson, D.V.M., Ph.D., Principal Investigator

Change from: Ronald G. Menton, Ph.D., Study Statistician

Change to: Nancy A. Niemuth, M.S., Study Statistician

Reason for Change: Position and title changes for responsible individuals on this study.

Impact on study: None.

Change No. 2: Page 12, Section V.D.1.e.

Change first sentence from: Relative humidity -- Maintained at 40-70 percent.

Change to: Relative humidity -- Maintained at 30-70 percent.

Reason for Change: The 1996 Guide states that relative humidity should be maintained
in this expanded range.

Impact on Study: None.

Change No. 3: Page 22, Protocol Amendment No. 2, Change No. 2

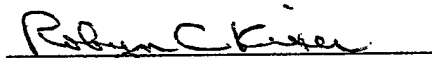
Change second sentence from: Neutral buffered formalin specimens will be evaluated for the presence or absence of histopathologic markers.

Change and add to second sentence: Neutral buffered formalin-fixed specimen from all HD-exposed ears of the HD-control and all treatment groups will be evaluated for the presence or absence of histopathologic markers. Control ears (i.e., not exposed to HD and/or to compound) will not be evaluated routinely for histopathology and will be considered to be without pathology (i.e., scored 0). If there appears to be an effect of a compound on the REW, or histopathology results for the right ear specimen show a statistically significant difference, then the corresponding left ear will be evaluated and a score assigned.

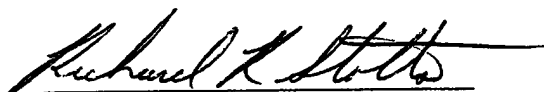
Reason for Change: To eliminate the time required for evaluating left ears except in cases where candidate compound activity is noted.

Impact on Study: This change provides for a more efficient and timely evaluation process.


Approved:


Robyn C. Kiser, B.S., RLATG
Study Director

12-14-98
Date


LTC Richard R. Stotts, COR
USAMRICD

14 Dec 98
Date


Elisha N. Morrison, M.S.
Senior QA Specialist

12/15/98
Date

APPENDIX B

Tables

Table B-1. Efficacious Task 95-43 Compounds

ICD #	Compound Name	Compound Class	Pre(-) or Post (+) Treatment Time (min)	Vehicle	Dose (mg)	Tissue Harvest Time (hr)	Mean REW ¹ Statistically Less Than Controls	Incidence of Microvesication Statistically Less Than Controls	Severity of Microvesication Statistically Less Than Controls	Incidence of Epidermal Necrosis Statistically Less Than Controls	Severity of Epidermal Necrosis Statistically Less Than Controls	Incidence of CEN ² Statistically Less Than Controls	Severity of CEN ² Statistically Less Than Controls
1304	2-Mercaptopyrindine-1-oxide	HD scavenger	-15	Ethanol	0.6	24	X						X
1307	6-Methyl-2-Mercaptopyrindine-1-oxide	HD scavenger	-15	Acetone	0.6	24	X						X
1308	4-Methyl-2-Mercaptopyrindine-1-oxide	HD scavenger	-15	Ethanol	0.6	24	X					X	X
1316	II-134	Protease inhibitor	-15	Ethanol	0.6	24	X						
1471	NA	Protease inhibitor	-60	Ethanol	1	24	X						
1541	NA	PADPRP inhibitor	-30	Ethanol	0.6	24	X						
1548	NA	PADPRP inhibitor	-30	DMSO	0.6	24						X	X
1579	NA	Protease inhibitor	-60	Ethanol	0.6	24	X		X				
1619	Thiouraine sodium salt	HD scavenger	-15	DMSO	0.6	24		X					
1700	C-8138	Anti-inflammatory	-15	Ethanol	0.6	24	X						
1756	NA	Protease inhibitor	-60	DMSO	0.6	24							X
1796	Benzoylene urea	PADPRP inhibitor	-30	DMSO	0.6	24							X
1861	NA	HD scavenger	-15	Ethanol	0.6	24							X
1880	NA	HD scavenger	-15	Acetone	0.4	24							X
1883	NA	Protease inhibitor	-60	DMSO	0.6	24							X
1915	NA	HD scavenger	-15	Acetone	0.6	24	X						X
1917	NA	Protease inhibitor	-60	DMSO	0.6	24							X
1918	NA	Protease inhibitor	-60	DMSO	0.6	24							X
1976	NA	Protease inhibitor	-60	Ethanol	0.6	24					X		
1984	NA	PADPRP inhibitor	-30	Ethanol	0.24	24	X						

Table B-1. Efficacious Task 95-43 Compounds
(Continued)

ICD #	Compound Name	Compound Class	Pre(-) or Post (+) Treatment Time (min)	Vehicle	Dose (mg)	Tissue Harvest Time (hr)	Mean REW ¹ Statistically Less Than Controls	Incidence of Microvesication Statistically Less Than Controls	Severity of Microvesication Statistically Less Than Controls	Incidence of Epidermal Necrosis Statistically Less Than Controls	Severity of Epidermal Necrosis Statistically Less Than Controls	Incidence of CEN ² Statistically Less Than Controls	Severity of CEN ² Statistically Less Than Controls
1986	NA	PADPRP inhibitor	-30	Ethanol	0.6	24				X			
1991	NA	PADPRP inhibitor	-30	Ethanol	0.6	24	X						
1992	NA	PADPRP inhibitor	-30	Ethanol	0.6	24	X						
2000	GP 10	Protease inhibitor	-60	DMSO	0.15	24		X					
2040	F-4765	Anti-inflammatory	-15	Methanol	0.6	24							X
2059	4-Amino-1-Naphthol Hydrochloride Tech	MADPRT inhibitor	-30	DMSO	0.3	24		X	X				X
2082	2198	Anti-inflammatory	-15	Ethanol	0.3	24	X						
2086	Indomethacin	Anti-inflammatory	-15	Ethanol	1	24	X	X	X		X	X	
2105	NA	PADPRP inhibitor	-30	Ethanol	0.6	24					X		
2121	NA	PADPRP inhibitor	-30	Acetone	0.6	24	X						
2126	NA	PADPRP inhibitor	-30	Ethanol	0.6	24							X
2130	NA	PADPRP inhibitor	-30	Ethanol	0.6	24							X
2136	4-Aminotetrafluorobenzamide	PADPRP inhibitor	-30	Ethanol	0.6	24		X	X				
2139	2,4-Dichloro-3,5-Dinitrobenzamide	PADPRP inhibitor	-30	Acetone	0.6	24		X					
2146	NA	PADPRP inhibitor	-30	DMSO	0.2	24							X
2232	NA	Protease inhibitor	-60	Acetone	0.6	24					X		
2437	NA	Protease inhibitor	-60	DMSO	0.6	24							X
2635	NA	Protease inhibitor	-60	Acetone	0.6	24					X		X
2669	NA	Protease inhibitor	-60	DMSO	0.6	24							X
2723	n-vanillyloleamide	Anti-inflammatory	-15	Ethanol	0.25	24	X	X	X		X	X	X

Table B-1. Efficacious Task 95-43 Compounds
(Continued)

ICD #	Compound Name	Compound Class	Pre(-) or Post (+) Treatment Time (min)	Vehicle	Dose (mg)	Tissue Harvest Time (hr)	Mean REW ¹ Statistically Less Than Controls	Incidence of Microvesication Statistically Less Than Controls	Severity of Microvesication Statistically Less Than Controls	Incidence of Epidermal Necrosis Statistically Less Than Controls	Severity of Epidermal Necrosis Statistically Less Than Controls	Incidence of CEN ² Statistically Less Than Controls	Severity of CEN ² Statistically Less Than Controls
2744	NA	Protease inhibitor	-60	Acetone	0.6	24	X						
2754	NA	Protease inhibitor	-60	Acetone	0.6	24	X						
2780	NA	Protease inhibitor	-60	Acetone	0.6	24	X				X		X
2783	NA	Protease inhibitor	-60	Acetone	0.6	24	X						
2786	NA	Protease inhibitor	-60	Ethanol	0.6	24	X						
2812	NA	Protease inhibitor	-60	DMSO	0.6	24							X
2827	CDB	Anti-inflammatory	-60	Ethanol	1	24	X						
2828	Stat One, hydrogen peroxide gel, 3%	Other	-120 and -30	NA	3	24							X
2842	Hydrocortisone	Anti-inflammatory	-120	Ethanol	0.24	24	X						
2844	NA	Anti-inflammatory	-120	Ethanol	0.24	24	X						
2845	Dexamethasone	Anti-inflammatory	-120	Ethanol	0.5	24					X		
2974	NA	Anti-inflammatory	-15	Ethanol	0.6	24	X						X
2975	NA	Anti-inflammatory	-15	DMSO	15	24							X
2976	NA	Anti-inflammatory	-15	Ethanol	0.6	24	X					X	X
2977	NA	Anti-inflammatory	-15	Ethanol	0.6	24						X	X
2980	NA	Anti-inflammatory	-15	Ethanol	0.6	24	X					X	X
2999	Ichthammol ointment	Anti-inflammatory	+120	NA	10	24	X						
3001	Sodium 2-Mercaptoethanesulfonate	Other	-15	DMSO	0.6	24		X					
3010	Ketoprofen	Anti-inflammatory	-15	Acetone	0.6	24	X						
3011	Sulindac	Anti-inflammatory	-15	Acetone	0.6	24	X						

Table B-1. Efficacious Task 95-43 Compounds
(Continued)

ICD #	Compound Name	Compound Class	Pre(-) or Post (+) Treatment Time (min)	Vehicle	Dose (mg)	Tissue Harvest Time (hr)	Mean REW ¹ Statistically Less Than Controls	Incidence of Microvesication Statistically Less Than Controls	Severity of Microvesication Statistically Less Than Controls	Incidence of Epidermal Necrosis Statistically Less Than Controls	Severity of Epidermal Necrosis Statistically Less Than Controls	Incidence of CEN ² Statistically Less Than Controls	Severity of CEN ² Statistically Less Than Controls
3102	NA	Protease inhibitor	-60	Acetone	0.6	24	X						
3105	NA	Protease inhibitor	-60	Acetone	0.6	24	X						
3138	Thiavitamin E Disulfide	HD scavenger	-15	Acetone	0.4	24	X						
3195	Sodium 3-sulfonatopropyl glutathionyl disulfide	HD scavenger	-15	DMSO	0.6	24			X				X

1 = Relative Ear Weight

2 = Contralateral Epidermal Necrosis

Table B-2. Results of Additional Testing of Efficacious Task 95-43 Compounds

ICD #	Compound Name	Compound Class	Pre(-) or Post (+) Treatment Time (min)	Vehicle	Dose (mg)	Tissue Harvest Time (hr)	Mean REW ¹ Statistically Less Than Controls	Incidence of Microvesication Statistically Less Than Controls	Severity of Microvesication Statistically Less Than Controls	Incidence of Epidermal Necrosis Statistically Less Than Controls	Severity of Epidermal Necrosis Statistically Less Than Controls	Incidence of CEN ² Statistically Less Than Controls	Severity of CEN ² Statistically Less Than Controls
1304	2-Mercaptopyrindine-1-oxide	HD scavenger	+10	Ethanol	0.6	24	X	X			X		X
1307	6-Methyl-2-Mercaptopyrindine-1-oxide	HD scavenger	+10	Acetone	0.6	24	X						X
1308	4-Methyl-2-Mercaptopyrindine-1-oxide	HD scavenger	+10	Ethanol	0.6	24	X	X			X	X	X
2086	Indomethacin	Anti-inflammatory	+10	Ethanol	1	24	X						
2086	Indomethacin	Anti-inflammatory	-15	Ethanol	1	48	X						
2723	n-vanillyloleamide	Anti-inflammatory	+10	Ethanol	0.25	24	X					X	X
2723	n-vanillyloleamide	Anti-inflammatory	-15	Ethanol	0.25	48	X				X	X	X
2723	n-vanillyloleamide	Anti-inflammatory	-15	Ethanol	0.25	72							X
2842	Hydrocortisone	Anti-inflammatory	-120	Ethanol	0.24	48	X						

1 = Relative Ear Weight

2 = Contralateral Epidermal Necrosis